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The cell cycle is regulated by the action of a family of cyclin dependent kinases (Cdks) which catalyze particular cell cycle transitions. Cdks are positively regulated through interaction with cyclins and are negatively regulated through phosphorylation and through association with inhibitory proteins of the CIP/KIP and INK4 families. Our research has focused on the role of p57KIP2 in development and cancer. We have found that p57KIP2 is expressed in a highly cell type specific manner during embryonic development and in adult tussues, being most highly expressed in terminally differentiated cells. Through analysis of p57KIP2 deficient mice, however, we have found that p57KIP2 is required for normal development of several tissues including the kidney, lens, muscle, and bone. We also discovered the p57 is involved in the human overgrowth and cancer predisposition disease Bechwith-Weidemann Syndroem, BWS. Current studies are aimed at understanding in greater detail the function of p57KIP2 and its possible role in human cancers.

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S Elledge

PI - Signature Date

Table of Contents

Introduction

The eukaryotic cell cycle is regulated primarily at two points, in G1 prior to entry into S-phase and in G2 prior to entry into mitosis. The commitment to a round of cell division is made at a point in G1, referred to as the restriction point in mammalian cells[1-4] or START in yeast[5]. Passage through the restriction point depends critically on mitogen signals, but once this point is passed, cells are committed to S-phase and the remainder of the cycle in a mitogen independent manner[4]. Passage through the restriction point is thought to be the primary event controlling cell proliferation. Therefore, elucidating how positively and negatively acting genes function to regulate the G1/S transition and how mutations in these genes disrupt normal cell cycle control has been a primary focus of cancer research. Central to this focus has been the investigation of the role of cyclin-dependent kinases (Cdk) in the control of cell proliferation.

Cyclins, Cdks, and positive growth control. Cdks are protein kinases that require association with cyclins and phosphorylation for activity[5-8]. Cyclins promote cell cycle transitions via their ability to associate with and activate their cognate Cdks[5-12]. Cyclins D and E function in G1[6, 10, 13-16], and overexpression of cyclin D1 or cyclin E shortens G1 and accelerates entry into S-phase[1, 3, 17, 18]. Amplification of cyclins, D1, D2 and E have been identified in several tumors[19-23]. Cyclin D1 was identified as the PRAD1 oncogene [24]. Cyclin A was identified as the site of integration of HBV in a hepatocellular carcinoma[25]. Taken together, these observations suggest that inappropriate activation of Cdks is a mechanism that cells frequently use to reach the oncogenic state.

Cyclins D1,D2, and D3 bind Cdk4 and Cdk6 kinases and can phosphorylate and inactivate Rb[6, 26-29]. Because D-type cyclins are required for proliferation only if cells have an intact Rb gene, it is thought that Rb inactivation is their primary role. Cyclin E binds to and activates Cdk2 and considerable evidence has accumulated indicating that cyclin E/Cdk2 is the primary kinase involved in the G1/S transition[14, 15, 30-33]. In addition, a close homolog of Cdk2 - Cdk3 - is also thought to play a unique role in the G1/S transition[31]. Cyclin A binds Cdk2 and Cdc2 and is required for both S-phase and the G2/M transition [34-36], while cyclin B/Cdc2 complexes appear to be specific for control of mitotic entry.

Although Cdks are thought to be the critical regulators of cell proliferation, little is known about how cyclin/Cdk complexes regulate cell proliferation during development. In this regard, we have performed an analysis of the expression of the major cyclins during mouse embryonic development and in adult tissues. We have discovered that general cyclins E, A, B, and F are expressed in all proliferating tissues while the D cyclins are distributed in a pattern distinct for each cyclin but which is a subset of the general cyclins (Parker, Harper and Elledge, unpublished results). This is consistent with the notion that D cyclins are the primary initiators of cell cycle entry and orchestrate development. We have recently observed that cyclin D1 is the only D-type cyclin induced when breast cells proliferate during pregnancy. We and our collaborators in the Weinberg laboratory discovered that development of the breast during pregnancy is dependent upon cyclin D1[37]. As mentioned above, amplification of D-type cyclins is frequently observed in breast cancer. This provides a link between development and cancer and indicates that the developmental history of the breast is relevant to its susceptibility to tumorigenisis.

Since the controls utilized during development to regulate cell proliferation are similar to those utilized in maintenance of the non-proliferative state in differentiated tissues, it is likely that these controls are reactivated or overcome in cancer. Another example of this comes from our observation that cyclin D1 is expressed at extremely high levels in the retina and is required for its development [37]. Presumably the inability to properly develop the retina in cyclin D1 mutants reflects an inability to overcome Rb. In what is clearly more than a coincidence, the retina is the same tissue in which high frequency tumors arise in Rb mutant humans. It is therefore likely that the Rb protein is important in both development of that tissue and its maintenance in the non-proliferative state. Our understanding of the links between development and cancer is in its infancy and is an area in which there is a great need to increase our knowledge base.

Tumor suppressor proteins and negative growth control. Rb and p53[29, 38] are the most well understood tumor suppressors. Mutations in these are found frequently in many human cancers[39,40], and reintroduction of wild-type genes into $p53^-$ or Rb^- tumor cells can suppress the neoplastic phenotype suggesting that loss of function of these genes contributes to tumorigenesis[28, 41, 42].

Mutations in p53 are the most common lesions observed in human malignancies, occurring in greater than 50% of all tumors[39] including those of the breast. The percentage is much higher if loss of p53 function via association with viral oncoproteins (E1B of adenovirus and E6 of papilloma virus) or amplification of the p53 binding protein MDM2 are included[43]. p53 deficient mice are prone to the spontaneous development of a variety of tumor types[44]. Cellular responses to DNA damage such as apoptosis and the G1 checkpoint are dependent on p53[45-53]. p53 also controls a spindle checkpoint and prevents genetic alterations such as gene amplification[54, 55]. p53 regulates the expression of $p21^{CIP1}$, an inhibitor of G1-cyclin/Cdks, in response to DNA damage[56-60]. Using a p21 knockout mouse, we have determined that p21 is required for full function of the G1 checkpoint in response to γ-irradiation, although there is residual checkpoint function[56]. Furthermore, these mice do not show the high rate of spontaneous tumor formation seen in p53-deficient mice. It is not clear whether p53's role in oncogenesis is through its checkpoint or apoptotic deficiencies, or a combination of these.

The current view of the role of Rb in the cell ccyle is that hypo-phosphorylated Rb functions during G1 in part to block the activity of E2F and related transcription factors that are required for the expression of genes involved in S-phase[29]. Hyper-phosphorylation of Rb or association with DNA tumor virus oncoproteins such as E1A results in release of E2F and is correlated with passage into S-phase.

The above observations are consistent with a model in which increased cyclin/Cdk activity in tumors, whether by increased cyclin expression or decreased negative regulation, can overcome the cell cycle repression function of Rb via direct phosphorylation and inactivation of its growth inhibitory function. Rb therefore acts as a potential energy barrier in the pathway that cyclin/Cdks must overcome to activate cell cycle entry. Removal of the barrier (Rb) may reduce the levels of kinase activity required, but some Cdk kinase activity is still required for the process of DNA replication and can therefore act as a target of further negative regulation. In this model, p53 acts to reduce the frequency of mutations that lead to altered growth control and to kill cells that have undergone extensive damage or are inappropriately growing. To fully understand this aspect of cancer, cell cycle dysfunction, it is imperative that we have a complete understanding of the regulation of cyclin dependent kinases and their regulators in the tissues of interest.

The cell cycle and development: potential roles for Cdk inhibitors. Once proliferation and morphogenesis have constructed a particular structure, it is of paramount importance that the proliferative state cease and be replaced with a homeostatic state. While much attention has been focused on how cells enter the cell cycle, little is known concerning the strategies organisms employ to exit the cycle and maintain the non-proliferative state. This state is of great importance to an organism because the vast majority of its cells exist in a non-proliferative state throughout adult life. The inability to appropriately halt growth can lead to malformation during development, and to cancer. Thus, equally important in the execution of developmental programs is the arrest of growth once the program is complete. While the control of terminal differentiation promises to be complex, cell cycle arrest via inactivation of Cdks is likely to be a central feature. Recently a new class of Cdk regulatory molecules have emerged that are potential mediators of cell cycle exit and maintenance of the non-proliferative state. These are the inhibitors of cyclin-dependent kinases, CKIs. Currently two structurally defined classes of CKIs exist in mammals that are exemplified by $p21^{CIP1}$ [57-60] and $p16^{INK4/MTS1}$ [61-65].

Cyclin-dependent kinase inhibitors: mediators of negative cell cycle control. Cdk inhibitory proteins are a group of proteins that associate with and inhibit Cdks. These versatile molecules have potential roles in cell cycle arrest, checkpoint function and development and are likely to cooperate with Rb, p53, and other negative regulators in maintaining the non-proliferative state throughout adult life. At the time of submission of this grant in December 1993, the first mammalian Cdk inhibitors $p21^{CIP1/WAF1}$ (57-60) and $p16^{INK4a}$ (61) had only recently been identified. Subsequently, we and others identified additional inhibitors including p27, p57, p15, p18, and p19 (refs 61-69). We identified $p21^{CIP1}$ in a two-hybrid screen designed to identify proteins that associate with Cdk2 (57). Importantly, this protein was simultaneously cloned by several other laboratories. p21 was cloned as a p53 activated gene by the Vogelstein laboratory (59), as a Cdk associated protein by the Beach laboratory (58), and as an S-phase inhibitory cDNA in senescent cells (60). Since then we and others have identified two other members of the p21 family, p27 and p57. p57, also known as KIP2 has been the focus of this study. It is expressed in the breast and is localized to 11p15.5m a locus involved in breast cancer (see below).

Involvement of 11p15.5, the location of KIP2, in human cancers including cancer of the breast. Several chromosomal regions show frequent loss of heterozygosity(LOH) in breast tumors including but not exclusively 3p, 7q31, 11p15, 11q13 and 17p(reviewed in 96). The chromosomal location of KIP2, 11p15.5, marks it as a candidate tumor suppressor gene of the breast. The involvement of 11p15 in the breast is well documented(113-119). 35% of breast tumors show LOH at 11p15.5(119) and this LOH is associated with poor prognosis(119). Furthermore, 11p15 LOH has been associated with metastasis(116) and there is evidence that 2 distinct breast tumor suppressor genes may reside at this locus(118). 11p15 has also been intensively investigated because of frequent LOH at this locus in a number of other human cancers including bladder, lung, ovarian, kidney, and testicular carcinomas (reviewed in 70). Several childhood tumors including Wilms' tumor, adrenocortical carcinoma, rhabdomyosarcoma, and hepatocellular carcinoma show specific loss of maternal 11p15 alleles, suggesting a role for genomic imprinting. Chromosome transfer experiments have also indicated a tumor suppressor gene resides at this locus, the WT2 gene involved in Wilms' tumor and possibly rhabdomyosarcoma (reviewed in 71), either of which could be due to loss of a Cdk inhibitor. In addition, rearrangements in the 11p15 region are found in Beckwith-Wiedemann Syndrome (BWS) which is characterized by numerous growth abnormalities, including macroglossia (enlarged tongue), gigantism, visceromegely (enlarged organs) and an increased risk (7.5%) of childhood tumors (72). BWS occurs with an incidence of 1 in 13,700 births, 85% of which are sporadic and 15% familial(73). Genetic analysis indicates maternal carriers, also suggesting a role for genomic imprinting(reviewed in 74). Several features of KIP2 make it a reasonable candidate as a mediator of some phenotypes of BWS. First, a Cdk inhibitor could explain both overgrowth and tumorigenesis phenotypes. Furthermore, the expression pattern of KIP2 in mouse correlates with areas known to be affected in BWS including the tongue, kidney, muscle, and the eye. Third, KIP2 is imprinted and maternally expressed. Furthermore, LOH at 11p15 in Wilms' tumors are exclusively maternal, offering further support for the possibility that KIP2 might be the WT2 gene. LOH of the breast has not yet been examined for parental specificity of LOH. However, the potential for the existence of two tightly linked tumor suppressors(75) affecting the breast at 11p15 might complicate the analysis of parentally biased LOH depending on the relative frequency of the two events. Nevertheless, the biochemical properties of KIP2, its physical location and expression patterns suggest that it may be the tumor suppressor at 11p15.

The goals of our work were: 1) to determine whether p57 is imprinted in the breast, 2) to construct mice lacking p57, 3) to analyze the phenotype of mice lacking p57, 4) to analyze the role of the QT domain in p57 function by looking for binding proteins, and 5) to characterize the regulation of p57 and 6) to look for additional CKIs in the breast. To date, we have made significant progress on these initial goals. Our progress in these areas is summarized below.

Body

Aim 1: Determination of p57 imprinting status in the breast.
We have completed this Aim and described this in last years report..

Aim 2: Construction of mice deficient in KIP2.. We completeld this aim and discussed it in last years report.

Aim 3: Analysis of p57 mutants animals.
We have completed an exhaustive analysis of the mutant phenotypes present in the p57 mutant animals last year. In the past year we have investigated the possible overlap between CKIs by making mice mutant for both p57 and p27. p27 has been implicated in growth control and possibly cancer. The phenotypes of mice lacking these two inhibitors are discussed below in the order in which we detected them.

A. Enhanced embryonic lethality in p27/p57 double mutants. To generate mice mutant for both of these *CKIs*., male $p27^{-/-}p57^{+/+}$ and female $p27^{+/-}p57^{+/-p}$ (p denoting paternal origin of the mutant *p57* allele) mice were mated to enrich for double mutants. Since the $p57^{KIP2}$ gene is imprinted, only the allele inherited from the mother (m denoting maternal origin) need be mutant in order to produce phenotypically null offspring[76]. As shown in Table 1, no *p57*-deficient animals survived to the time of genotyping (10 days of age), irrespective of the status of *p27*, confirming our earlier finding that *p57* is essential for neonatal survival[76]. Dead pups found in newborn litters were shown to have a $p57^{+/-m}$ genotype. Although expected frequencies of mice with all possible genotypes were found in embryos harvested from E13.5-E18.5 (Table 1), we observed a significant incidence of embryonic lethality in *p57* mutant embryos, as has been reported[76, 77]. Interestingly, deletion of *p27* significantly increased the frequency of embryonic lethality by a factor of two when the viability of $p27^{-/-}p57^{+/-m}$ animals are compared to that of $p27^{+/-}p57^{+/-m}$ animals (Table 1). However, we were unable to make a valid comparison between the lethality of $p27^{-/-}p57^{+/-m}$ (20% lethality) and $p57^{+/-m}$ animals from our original report (10% lethality[76]), because of different genetic backgrounds. Embryos died over a wide window between E12 to E16.5 and embryos that were alive at the time of harvesting showed heterogeneous degrees of growth retardation indicative of intermediate penetrance, likely resulted from the nature of mixed genetic backgrounds among these animals. Histopathological examination of mutant embryos failed to show defects in the cardiovascular system or erythropoiesis, common sources of embryonic lethality. However, defects were observed in the placenta of mutant animals, an organ critical for fetal development and survival.

Of the several types of placentas, mice primarily have hemochorial placenta where maternal blood is no longer contained in blood vessels but is in direct contact with fetal trophoblasts which also embed fetal capillaries in the labyrinth zone. Characteristics of hemoendothelial placenta are also found in mice where maternal blood is in direct contact with the fetal capillary endothelium. In placentas derived from $p57^{+/-m}$ single or $p27^{-/-}p57^{+/-m}$ double mutants, the labyrinth zone was less vascularized and contained more trophoblasts than those from wildtype or $p27^{-/-}$ mice (Fig. 1A, compare a and b). The diameter of most mutant fetal capillaries was reduced to the size of a single fetal red blood cell, leading to the appearance of less vascularization. Normal placentas contain numerous open spaces (the fetal capillary and maternal blood sinus) that are replaced with trophoblasts in the mutant. We have found this phenotype varies considerably, ranging from very little vascularization (Fig. 1A, b) to almost normal in those animals who survived to term (not shown), consistent with the variability in timing and rates of embryonic lethality. The degree of placental impairment correlates with size of the embryo, with more developmentally defective placentas containing smaller embryos.

In addition to reduced vascularization, placentas from *p57* mutant mice regardless of the status of *p27* contain areas that are marked by hyaline membranes in the labyrinth zone (Fig. 1A, c, and data not shown). Necrosis was observed in these areas and is likely to be due to blockade of the blood supply by hyaline membranes. Hyaline membranes are formed in response to endothelium damage, as has been observed in the respiratory distress syndrome caused by capillary or alveolar epithelium damage[78]. Given the biochemical functions of *p27* and *p57* and their roles in other tissues, we suspected that the absence of these *CKIs* might alter the differentiation of trophoblasts in the labyrinth zone, allowing them to proliferate inappropriately. This would result in limited space available for the fetal capillaries and maternal blood sinus, possibly leading to blood vessel damage and the formation of hyaline membranes. Indeed, BrdU incorporation assays demonstrated increased proliferation in $p27^{-/-}p57^{+/-m}$ mutant placentas (Fig. 1C, compare a and b). These assays were performed on a litter harvested at E18.5 when the normal placenta had already ceased proliferation in order to more easily observe the proliferation defects due to inhibitor loss. The fraction of BrdU positive cells was greatly increased in placentas from $p27^{-/-}p57^{+/-m}$ mice relative to $p27^{+/-}$ and $p27^{-/-}$ placentas (25 and 10-fold respectively) and was significantly larger (4-fold) than that found with $p27^{+/-}p57^{+/-m}$ placentas (Fig. 1C). There was considerable heterogeneity in the placental phenotypes of mutant animals. As shown in Table 1, a significant percentage of the $p27^{-/-}p57^{+/-m}$ embryos escape embryonic lethality and placentas from these animals appear to be much less defective than those shown here. Furthermore, while the additional loss of *p27* increases proliferation rates, it does not significantly exacerbate the histological defects observed in *p57* mutant placentas. It does, however, change the penetrance of the placental failure phenotype, making the placenta twice as likely to fail. *p57* is highly expressed in the labyrinth zone but not the adjacent spongiotrophoblast zone (Fig. 1B, a). All of the placental defects are observed exclusively in the labyrinth zone, while other aspects of the placentas from these mutant mice are normal (not shown). *p27* is expressed both in labyrinth and spongiotrophoblast zone (Fig. 1B, c). This concordance of expression suggests that the defects observed are cell autonomous and indicates that *p27* can provide some compensatory function in the labyrinth zone in the absence of *p57*. Thus, both *p27* and *p57* are expressed in the tissue found defective in the mutant embryos suggesting that the phenotype is very likely to be cell autonomous.

Embryos from these crosses that were not affected by placental defects were examined for developmental phenotypes, including those previously reported for the $p57^{+/-m}$ mutant. All affected tissues displayed phenotypes equivalent to those seen in *p57*-deficient embryos[76] with the exception of the lens where a profound defect was observed in the $p27^{-/-}p57^{+/-m}$ double mutants as described below.

B. Dramatic defects in lens development of p27/p57 double mutants. Ocular lens development involves several steps. By embryonic day 11.5, a sphere of epithelial cells have formed the lens vesicle. At this stage, cells in the posterior region undergo cell cycle exit and begin to elongate toward the anterior wall. Three days later, elongation is complete and these differentiated fiber cells are capped on the anterior wall by a layer of immature epithelial cells. These cells proliferate and migrate to the equatorial zone where they exit the cell cycle and differentiate to form secondary lens fiber cells[79]. Previously, we demonstrated that *p57* is required for appropriate cell cycle exit in the lens. Cells in the equatorial zone express high levels of *p57* (ref. 76 and Fig. 2D) and loss of *p57* allows these cells to continue to proliferate temporarily[76]. However, *p57*-deficient lens cells eventually undergo cell cycle exit and differentiate into lens fiber cells. These lenses are relatively normal but in some genetic backgrounds accumulate vacuoles indicative of incomplete lens fiber cell elongation and or apotosis. The ability of *p57*-deficient lens cells to differentiate, albeit with reduced kinetics, implies the existence of a second regulatory pathway controlling cell cycle exit in this tissue.

Cell cycle exit could be achieved by downregulating cyclins or by inducing additional *CKIs*. To examine these possibilities, we performed *in situ* hybridization analysis to determine the

transcriptional status of *CKIs* and D-type cyclins in the lens during differentiation. At day E15.5, all three D-type cyclin mRNAs are expressed in the lens, with *D2* showing the strongest expression (Fig. 2A-C). *D2* and *D3* also show mRNA expression in the posterior chamber which contains primarily differentiated cells. *D1* and *D2* had been previously shown to be expressed at day E13.5 in cells of the anterior epithelia and equatorial zone, and *D2* expression was observed in the posterior chamber (Fromm and Overbeek, 1996), suggesting that additional inhibitory signals are likely to be required to counteract their growth promoting activities. Consistent with this notion, we observed expression of a second *CKI*, *p27*, in the same cells as *p57* in the equatorial zone (Fig. 2E) and in the posterior chamber of the lens. In contrast, *p21* transcripts were not detected in the lens (Fig. 2F).

Dramatic defects in lens development in p27/p57 double mutant mice are apparent as early as E13.5, a time at which posterior cells have normally already initiated elongation into primary lens fiber cells (Fig. 3D). It should be noted that phenotypically, $p27^{-/-}$ lenses were indistinguishable from wild type lenses (data not shown), and $p27^{+/-}p57^{+/-m}$ lenses were indistinguishable from lenses derived from $p57^{+/-m}$ mice (Fig. 3G-I). However, $p57^{+/-m}$ lens defects in the genetic background resulting from a cross with $p27^{-/-}$ mice are slightly more severe than those we previously observed (ref. 76 and Fig. 3G-I). Most striking is the finding that lens vesicles from double mutant mice are filled with nuclei as assessed histologically (Fig. 3F). Although cells adjacent to the posterior wall fail to elongate in the double mutant, this effect , albeit less dramatic, is also seen in this background in $p57^{+/-m}$ mice (Fig. 3E, F). By E15.5, the posterior zone nuclear density has increased further in double mutants while no nuclei are detected in wild type or $p27^{-/-}$ mutants, and far fewer nuclei are present in $p27^{+/-}p57^{+/-m}$ or $p57^{+/-m}$ lenses (Fig. 3A-C and Fig. 4A-C). The appearance of large numbers of nuclei in the lens fiber cell compartment is consistent with ectopic proliferation. To verify this supposition directly, *in situ* BrdU incorporation assays were performed. The posterior chamber in E13.5 and E15.5 $p27^{-/-}p57^{+/-m}$ lenses contained many more actively dividing cells than did those of $p57^{+/-m}$ and $p27^{-/+}$ $p57^{+/-m}$ heterozygous animals (Fig. 4D-F and data not shown). Sections from $p27^{-/-}p57^{+/-m}$ lenses at E15.5 displayed 66-fold more BrdU-positive cells than lenses from $p27^{+/-}$ $p57^{+/+}$ mice and 8-fold more than lenses from $p27^{+/-}p57^{+/-m}$ mice, and similar values were observed at E13.5 (Fig. 4P). We also note that *p57* mutant lenses (irrespective of *p27* status) are 15-20% larger than wild type lenses and cataracts were apparent (Fig. 3A-C and data not shown).

The appearance of large vacuoles in the anterior chamber of *p57* mutant lens (irrespective of *p27* status) could be a result of the failure of fiber cells to elongate, or a consequence of cell death by both apoptosis and necrosis, or both. Lens fiber cell elongation is a hallmark of differentiation and requires proper temporal and spatial expression of lens crystallin proteins, expression patterns of which serve as reliable markers of the lens differentiation program. Mice deficient for the transcription factor *SOX1* display defects in the differentiation of lens fiber cells as indicated by the absence of induction of γ-crystallins, leading to incomplete elongation and large vacuoles in the lens (Nishiguchi et al., 1998). $p57^{+/-m}$ lenses display substantially reduced levels of β- and γ-crystallins (Fig. 4H, K), compared to $p27^{-/-}$ lenses (Fig. 4G, J), and crystallin expression is reduced to undetectable levels when combined with *p27* deficiency (Fig. 4I, L). Control experiments (not shown) indicate that the staining seen in the region of the vacuoles in the anterior of the chamber is due to non-specific interactions (edge effect) of the secondary antibody used and does not reflect crystallin expression. These data indicate that *p57* and *p27* are required for proper lens fiber cell differentiation and elongation. To examine cell death, TUNEL assays were performed on E15.5 lenses. Apoptotic cells were detected in the posterior chamber of $p27^{+/-}p57^{+/-m}$ and $p27^{-/-}p57^{+/-m}$ mutant lenses, typically one apoptotic cell per 0.2 mm^2 cross section, but no apoptotic cells were detected in $p27^{-/-}$ lenses in this region (Fig. 4 M-O). Since there are approximately 100 cross sections per lens, there are approximately 100 apoptotic cells per lens compared to zero in a *p27* null lens. Histological evidence of necrosis was also found, and was most pronounced in regions immediately adjacent to vacuoles (data not shown). Thus, cell death may also contribute to vacuoles in *p57*-deficient lenses.

Aim 4. Analysis of the QT domain.
CKIs of the p21 family have multiple domains. For example, in addition to Cdks, p21 can bind PCNA and this interaction inhibits PCNA-dependent DNA replication[67, 68] making p21 a dual specificity inhibitor. Importantly, p21 can associate with PCNA and Cdks simultaneously and may serve to target active kinases to particular substrates. Human p57 also has multiple domains: An N-terminal Cdk-binding domain, a proline alanine-rich central domain called the PAPA repeat, and a C-terminal sequence, the QT domain, displaying 50% identity with the C-terminus of p27. In the mouse p57 gene, the central region has both a proline rich region and an acidic repeat region. With the exception of the inhibitory domain, the function of these additional domains are not known. However, the finding of strong conservation in the QT-domain with p27 suggests that the it has roles independent of Cdk binding or inhibition. One possible function for this domain is to recruit proteins to the cyclin/Cdk complex. We are planning to look for proteins that bind to C-terminus of p57 by the two hybrid system. We are also interested in over expressing that domain of p57 in transgenic animals. We have not initiated these studies yet.

Aim 5. Transcriptional control of $p57^{KIP2}$.
Through in situ analysis and immunohistochemistry we know where p57 is expressed and we described that data in last years report. We propose to perform an analysis of the regulatory sites in the p57 promoter that controls its expression. Toward this goal, we have generated transgeninc mice in which the promoter of p57 is fused to the lacZ gene. We found that 1kb of p57 promoter region is required to recapitulate the expression pattern in muscle and the lens. We are now in the process of further characterizing this piece of DNA and other regions of the promoter.

Aim 6. Identification of new CKIs and other potential regulators of Cdks from normal breast. This aim proposes to look for additional Cdk binding proteins in the breast using the two hybrid system and breast cDNA libraries. We have the cDNA libraries but have not begun the screens yet because we have been so busy analyzing the mice. Hopefully be can begin these interesting experiments during the next funding period.

Conclusion:

The last year was a very productive one for our lab and the cell cycle field in general. Our work funded under this grant allowed us to establish the role of $p57^{KIP2}$ in mouse development and the human cancer and overgrowth syndrome BWS. $p57^{KIP2}$ clearly acts as a regulator of cell proliferation in the adrenal gland, the lens epithelia, and certain chondrocytes. The partial dependency on $p57^{KIP2}$ for reducing cell proliferation reveals the redundant mechanisms used to limit tissue growth. A similar situation is observed in cell culture where agents that induce cell cycle arrest immediately increase levels of certain CKIs and subsequently reduce the levels of the cyclins and Cdks. While undergoing the process of reducing Cdk activity during differentiation, the absence of CKIs may allow additional cell cycles to occur before Cdk activity is sufficiently reduced to block cell cycle entry. In addition, other CKIs may provide Cdk inhibitory functions in the absence of $p57^{KIP2}$, as we have shown here in the lens development of p27/p57 double mutant mice.

CKIs are the ultimate effectors of signal transduction pathway intended to bring about cell cycle arrest and the patterns of expression during embryonic development suggest that particular *CKIs* play important roles in terminal differentiation in a tissue specific manner. However, the fact that mice lacking single *CKIs* display surprisingly few developmental phenotypes has brought into question the essential nature of *CKIs* for cell cycle arrest and differentiation. Our studies **funded by this grant demonstrate** that two *CKIs*, *p57* and *p27*, cooperate to control proliferation and differentiation in multiple tissues and reiterates the critical importance of *CKIs* to cell cycle control during development. The use of multiple *CKIs*, each controlled through distinct signaling pathways,

provides a flexible mechanism to control proliferation in a cell type specific manner. It is likely that the combinatorial use of *CKIs* will emerge as one of the principal means through which cell cycle arrest and differentiation are integrated during development.

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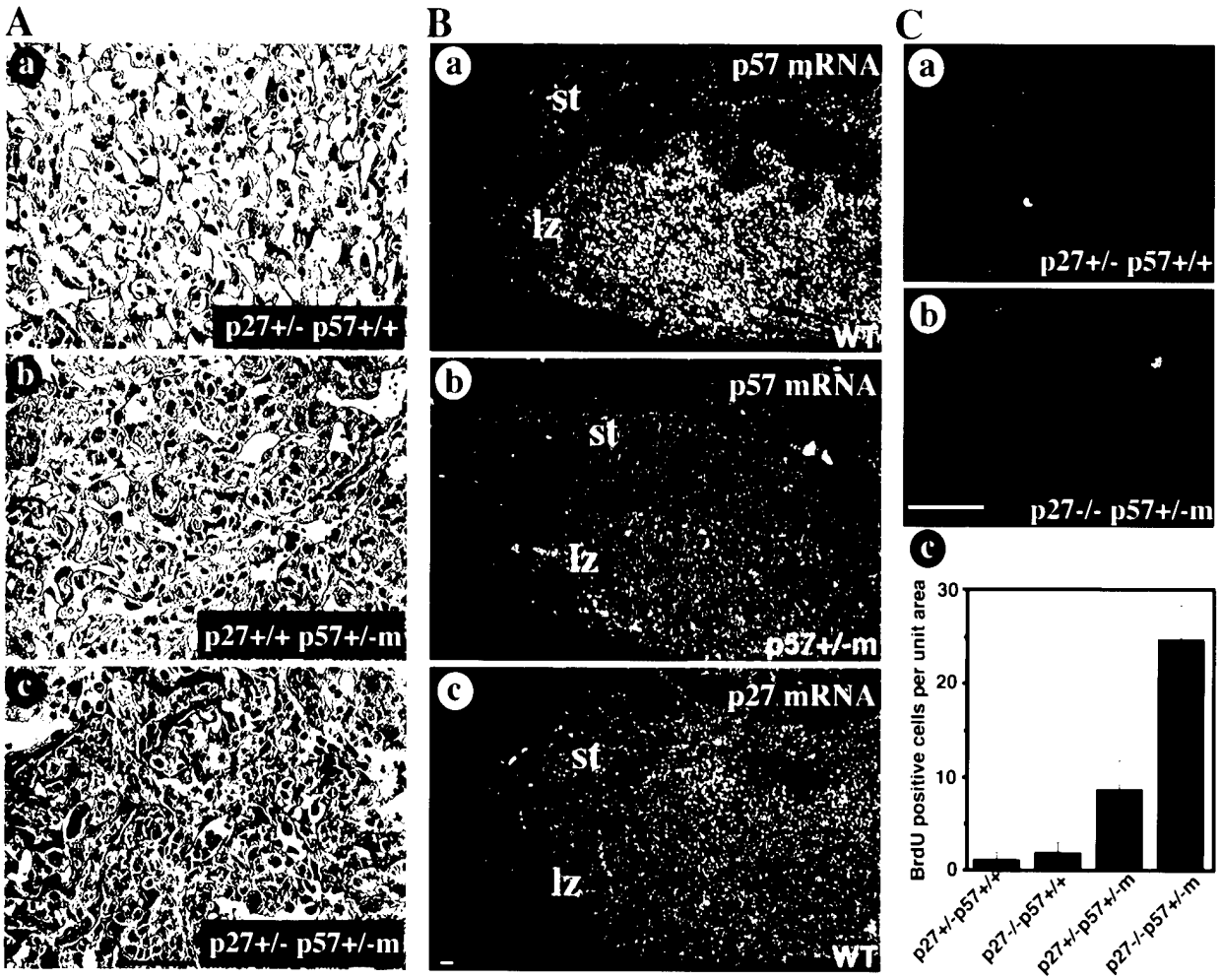

A
a
p27+/- p57+/+
b
p27+/+ p57+/-m
c
p27+/- p57+/-m
B
a
p57 mRNA
st
lz
WT
b
p57 mRNA
st
lz
p57+/-m
c
p27 mRNA
st
lz
WT
C
a
p27+/- p57+/+
b
p27-/- p57+/-m
c
BrdU positive cells per unit area
30
20
10
0
p27+/-p57+/+
p27-/-p57+/+
p27+/-p57+/-m
p27-/-p57+/-m

Appendix

Figure Legends

Figure 1 *p57* and *p27* are required for the proper development of the mouse hemochorial placenta. (A) Hematoxylin and eosin stained E12.5 placenta sections from (a) $p27^{+/-}$, (b) $p57^{+/-m}$, and (c) $p27^{+/-}p57^{+/-m}$ mice. Arrows in (c) indicate the hyaline membranes. (B) Expression of *p57*(a) and *p27*(c) in E12.5 placenta as detected by *in situ* hybridization. The specificity of the *p57* probe is demonstrated through the absence of signal in a placental section from a $p57^{+/-m}$ mouse (b). (C) BrdU incorporation assays reveal overproliferation in the labyrinth zone of $p27^{-/-}p57^{+/-m}$ placentas, as compared with $p27^{+/-}$, $p27^{-/-}$, or $p27^{+/-}p57^{+/-m}$ placentas. Placentas were harvested at E18.5. Quantitation of BrdU assays on embryos collected from a single litter are presented in (c). Error bars represent the standard deviation. lz, labyrinth zone; stz, spongiotrophoblasts zone. Scale bars, 200 μm.

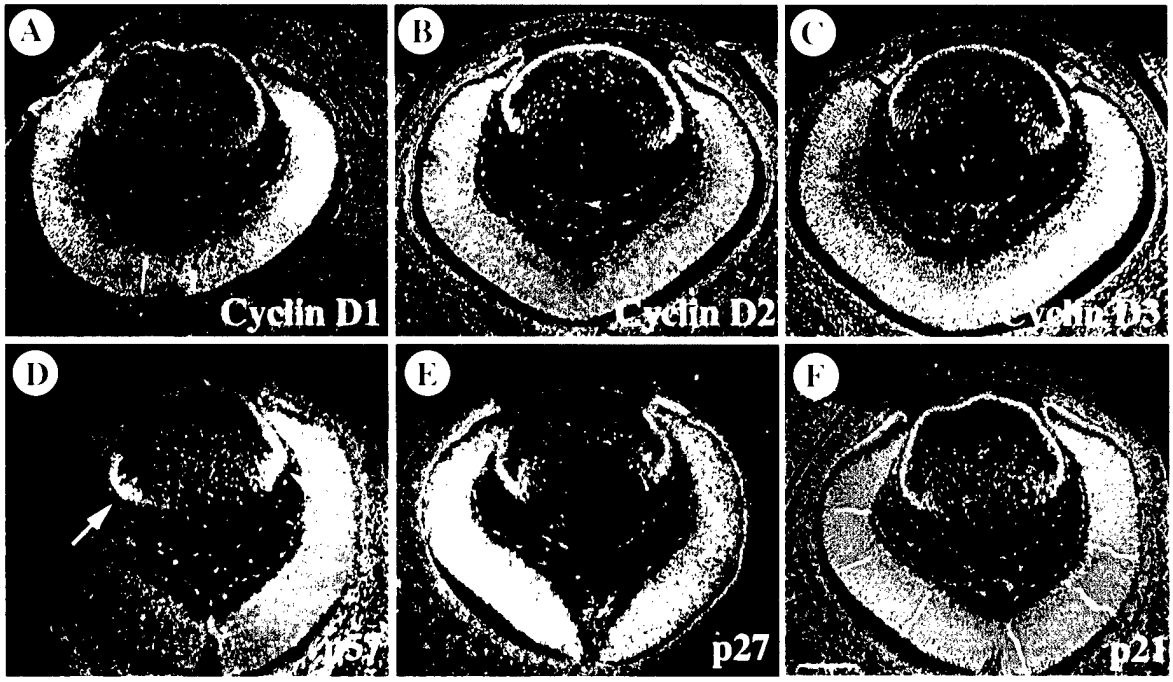

A
Cyclin D1
B
Cyclin D2
C
D
E
p27
F
p21

Figure 2 Expression of cell cycle regulatory genes during lens development. In situ hybridization was performed on transverse sections through the region of eye of an E15.5 embryo using antisense probes for cyclin *D1* (A), *D2* (B), *D3* (C), *p57* (D), *p27* (E), and *p21* (F). The arrow in (A) points to the pigmented epithelium (PE) which falsely stains positive for all probes because of the presence of pigmented-granules in these cells. Arrows in (D) indicate cells in the equatorial zone of the lens which express high levels of *p57*. *p27* is expressed in the equatorial zone and in the retina (r). Scale bar, 200 μm

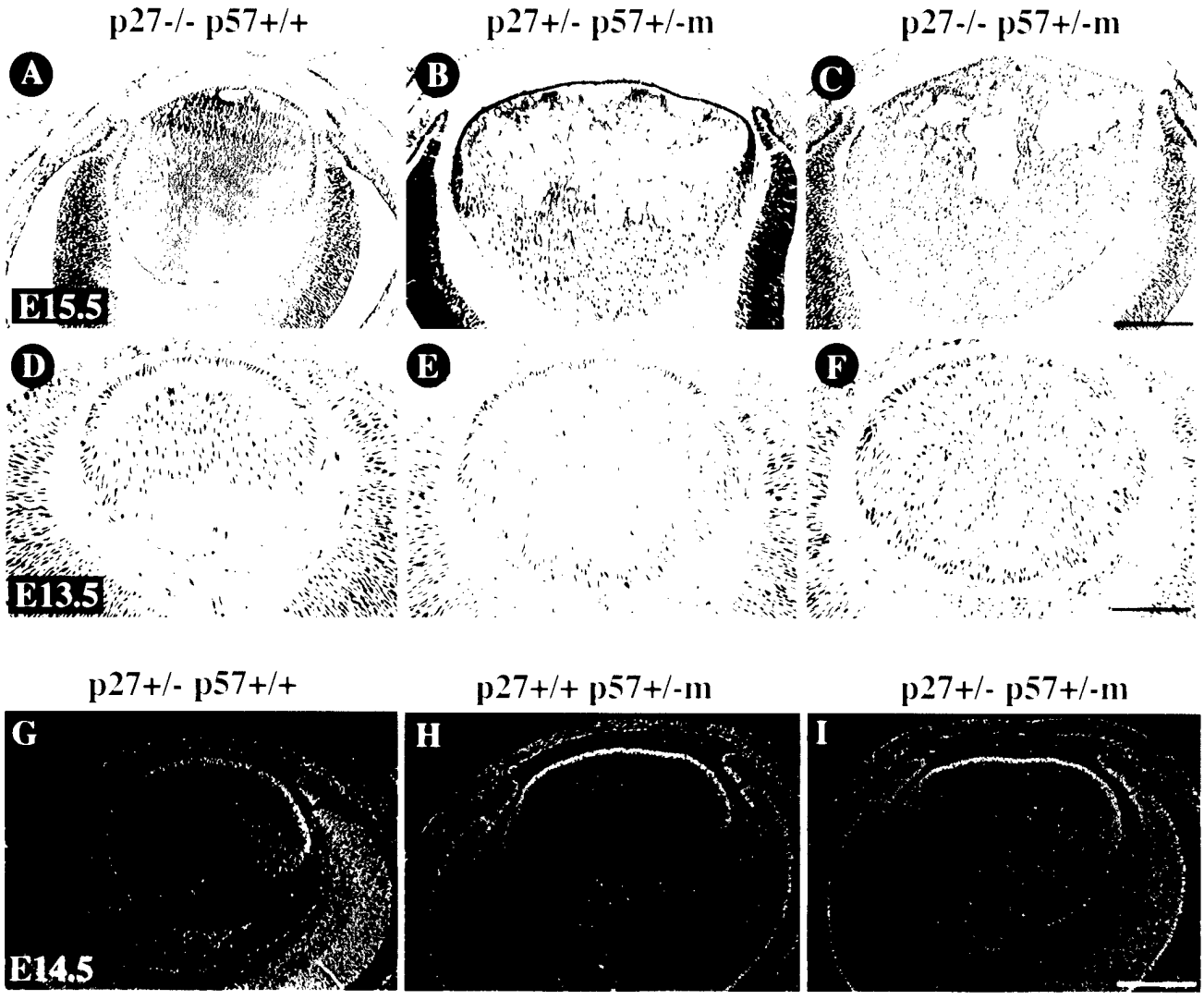
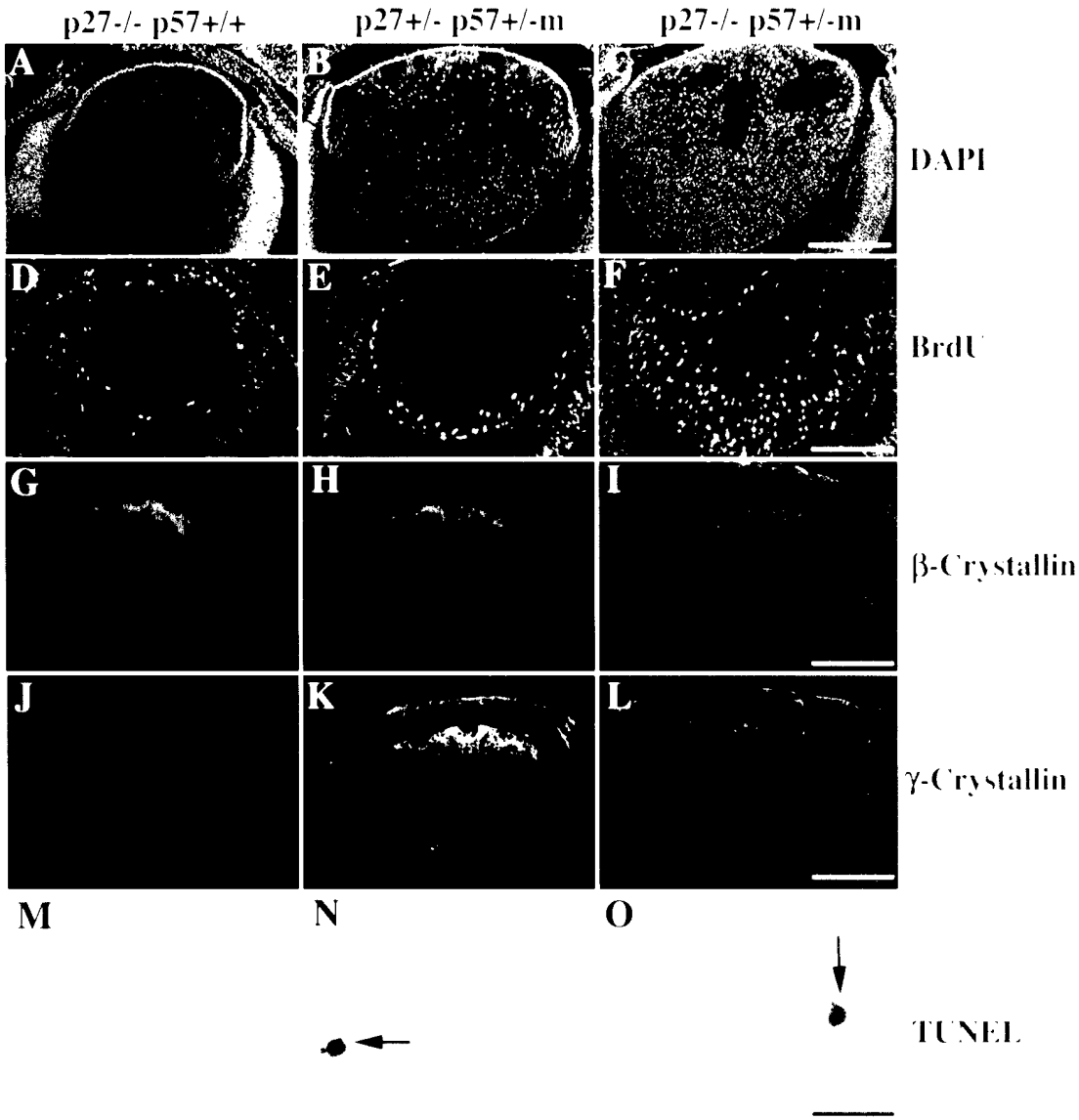

p27-/- p57+/+
p27+/- p57+/-m
p27-/- p57+/-m
A
B
C
E15.5
D
E
F
E13.5
p27+/- p57+/+
p27+/+ p57+/-m
p27+/- p57+/-m
G
H
I
E14.5

Figure 3 *p57* and *p27* are required for lens development. (A-C) Hematoxylin and eosin stained sections of E15.5 lenses from the indicated genotypes. (D-F) Hematoxylin and eosin stained sections of E13.5 lenses from the indicated genotypes. Nuclei at the posterior edge of $p57^{+/-m}$ (E) and $p27^{-/-}p57^{+/-m}$ (F) lenses are indicated by the arrow. (G-I) Nuclei in lens sections derived from E14.5 mice revealed by DAPI staining. Scale bar, 200 μm.


p27-/- p57+/+
p27+/- p57+/-m
p27-/- p57+/-m
A
B
C
DAPI
D
E
F
BrdU
G
H
I
β-Crystallin
J
K
L
γ-Crystallin
M
N
O
TUNEL

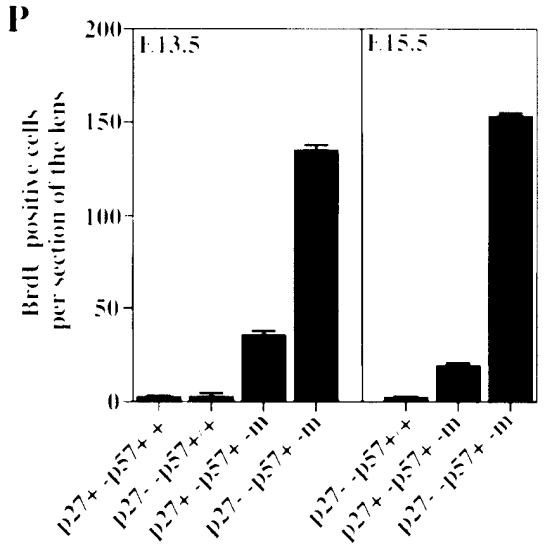

P
E13.5
E15.5
BrdU positive cells per section of the lens
200
150
100
50
0
p27+ -p57+ +
p27- -p57+ +
p27+ -p57+ -m
p27- -p57+ -m
p27- -p57+ +
p27+ -p57+ -m
p27- -p57+ -m

Figure 4 Overproliferation of lens fiber cells in *p27/p57* double mutants leads to compromised differentiation and increased apoptosis. (A-C) Nuclei of lens sections derived from E15.5 embryos revealed by DAPI staining. (D-F) BrdU incorporation assays demonstrate defects in cell cycle exit in $p27^{-/-}p57^{+/-m}$ lenses. (G-L) Immunofluorescence staining of β- and γ-crystallins demonstrates reduced expression of these two differentiation markers in the lens of $p57^{+/-m}$ mice (H, K) and the absence of expression in the lens of $p27^{-/-}p57^{+/-m}$ mice (I, L). (M-O) TUNEL assays detect apoptotic cells (arrow) in both $p57^{+/-m}$ and $p27^{-/-}p57^{+/-m}$ lenses. (P) Quantitation of BrdU incorporation assays at E13.5 and E15.5. BrdU-positive nuclei from a total of 6 sections for each genotype were determined and the average is shown in the histogram along with the standard deviation. Scale bars in (C), (F), (I), (L), 200 μm; in (O), 50 μm.

Table 1. Viability of mice lacking *p27* and *p57*.

Male $p27^{-/-}\ p57^{+/+}$ X Female $p27^{+/-}\ p57^{+/-p}$

Genotype	$p27^{+/-}\ p57^{+/+}$	$p27^{-/-}\ p57^{+/+}$	$p27^{+/-}\ p57^{+/-m}$	$p27^{-/-}\ p57^{+/-m}$
Number of offspring	19	25	0	0
Observed (%)	43	57	0	0
Expected (%)	25	25	25	25
Number of embryos	28	35	33 (7)[a]	27 (12)[a]
Observed (%)	23	28	27	22
Expected (%)	25	25	25	25
Lethality (%)	0	0	21	44

a. number in parentheses indicates dead embryos harvested between 13.5 and 18.5.

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Publications

Zhang, P., Wong, C., DePinho, R.A., Harper, J.W., and Elledge, S. J.(1998). Cooperation between the *Cdk* Inhibitors *p27KIP1* and *p57KIP2* in the control of tissue growth and development. *Genes Dev.* (in press).

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